Kinetics of inter-domain electron transfer in flavocytochrome cellobiose dehydrogenase from the white-rot fungus *Phanerochaete chrysosporium*

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The pre-steady-state kinetics of inter-domain electron transfer in the extracellular flavocytochrome cellobiose dehydrogenase from *Phanerochaete chrysosporium* was studied using various values of pH and substrate concentration. Monitoring at the isosbestic point of each prosthetic group indicated that the reductive half-reactions of flavin and haem were biphasic and monophasic respectively. When the observed rates of the flavin and haem reactions were plotted against substrate concentration, the behaviour of the second phase of the flavin reduction was almost identical with that of haem reduction at all substrate concentrations and pH values tested, suggesting that the formation of flavin semiquinone and haem reduction involve the same electron transfer reaction. Although flavin reduction by cellobiose was observed in the range of pH 3.0–7.0, the velocity of the next electron transfer step decreased with increase of pH and was almost zero above pH 6.0. The second phase of flavin reduction and the haem reduction were inhibited similarly by high concentrations of the substrate, whereas the first phase of flavin reduction showed a hyperbolic relation to the cellobiose concentration. Increase in pH enhanced the substrate inhibition of haem reduction but not the initial flavin reduction. Moreover, the dissociation constant K_d of flavin reduction and the substrate inhibition constant K_i of haem reduction decreased similarly with an increase of pH. From these results, it is evident that binding of cellobiose to the active site inhibits electron transfer from flavin to haem.

Key words: cellulose degradation, flavin semiquinone, haem, stopped flow.

INTRODUCTION

The white-rot fungus *Phanerochaete chrysosporium* produces extracellular flavocytochrome cellobiose dehydrogenase (CDH; EC 1.1.99.18) in the course of cellulose degradation [1–4]. CDH is a monomeric enzyme which contains flavin (FAD) and b-type haem as prosthetic groups, and the molar ratio of flavin/haem is known to be 1:1 [5,6]. The flavin and haem moieties are located in the 55 and 35 kDa domains respectively [7], and these domains are connected by a Ser–Thr-rich long linker region [8–10]. CDH oxidizes the reducing-end groups of cellobiose and cellodextrins to the corresponding δ-lactones using many different electron acceptors. Initially CDH was identified as an oxidase, utilizing molecular oxygen as an electron acceptor [1]. However, Wilson et al. [11] noted that dichloroindophenol was reduced much more rapidly than molecular oxygen, and suggested that the true electron acceptor of CDH *in vivo* might be quinines. On the other hand, the results that ferriacetate, ferricyanide and ferricytochrome *c* were good electron acceptors for CDH, but not for its separated flavin domain, clearly suggest that the intact CDH is primarily an Fe(III) reductase [12,13].

The kinetics of the reductive half-reaction of CDH was first investigated by Jones and Wilson [14]. In their experiment, however, pre-steady-state reduction rates of the prosthetic groups were obtained at pH 6.0, even though the optimum redox reaction of CDH is at acidic pH (approx. 4.0). We subsequently measured the reduction rates of flavin and haem at two different pH values, and found that only the flavin reduction proceeded at pH 5.9, whereas both flavin and haem were reduced effectively at pH 4.2 [15]. In addition to the effect of pH, substrate (cellobiose) inhibition of steady-state reduction of cytochrome *c* by CDH has been reported [16]. In the present study, the reduction rates of flavin and haem were measured in more detail, and the effects of pH and substrate concentration on reaction of CDH are discussed.

MATERIALS AND METHODS

Materials

CDH (EC 1.1.99.18) was purified from the medium of cellulose-grown cultures of *P. chrysosporium* as described previously [17]. The protein concentration was determined spectrophotometrically using $\epsilon_{421} = 130\ mM^{-1} \cdot cm^{-1}$ for the oxidized and $\epsilon_{429} = 184\ mM^{-1} \cdot cm^{-1}$ for the reduced enzyme [18]. D-Cellobiose was purchased from ICN Biomedicals Inc. For the pH dependence study, 50 mM buffers were used as described previously [18].

Anaerobic titration

For anaerobic reduction of CDH, 1 ml of 17.5 μM enzyme solution was made anaerobic by sequential evacuation and re-equilibration with oxygen-free wet argon in a 1 cm optical length anaerobic cuvette with side arms. During titration, absorption spectra of CDH were recorded with a Hitachi UV–Visible spectrophotometer U-3210. The concentration of the sodium dithionite solution used for reduction was standardized by anaerobic titration of 5 μM FMN solution with the same dithionite solution. To evaluate the electron distribution between prosthetic groups, the isosbestic points of haem (449 nm) and flavin (562 nm) [14] were monitored during the titration.

Abbreviation used: CDH, cellobiose dehydrogenase.

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Pre-steady-state kinetic studies

The rapid spectral change of CDH after mixing with cellobiose was monitored using an Olis RSM-1000 rapid scanning monochromator (Olis Inc., Bogart, GA, U.S.A.) at 30 °C in 50 mM sodium acetate (pH 4.5). The concentrations of CDH and cellobiose after mixing were 5 and 500 μM respectively. For the determination of the reduction rates of prosthetic groups in CDH, a stopped-flow experiment was carried out using an Applied Photophysics SX-18MV kinetic spectrophotometer with a 1 cm observation path length. CDH (final concentration 5 μM) and cellobiose (final concentration 25–500 μM) were mixed in 50 mM buffers of pH 3.0–7.0 at 30 °C. Absorption changes at 449 and 562 nm were used to monitor flavin and haem reduction respectively [14,15]. The observed rate constants k_{obs} for flavin and haem reduction were then plotted against cellobiose concentration S to obtain the limiting rate constant k_{lim} and dissociation constant K_d [19]. For flavin reduction, the hyperbolic substrate concentration dependence was fitted to the equation

$$k_{obs} = k_{lim}S/(K_d + S) \quad (1)$$

To estimate k_{lim} for the second phase of flavin reduction and for haem reduction, eqn (2) containing the substrate inhibition constant K_i was used as described in a substrate inhibition study of flavocytochrome b_2 [20]:

$$k_{obs} = k_{lim}S/(K_d + S + S^2/K_i) \quad (2)$$

At least three measurements were performed to determine the rate constants, and DeltaGraph 4.0 (DeltaPoint Inc.) and KaleidaGraph (Synergy Software) were used for the fitting.

RESULTS

Anaerobic dithionite titration of CDH

Quantitative dithionite titration was performed to evaluate the redox properties of CDH, as shown in Figure 1. When dithionite solution was added, increasing absorption at 562 and 533 nm, the α and β bands of reduced b-type haem respectively [5,6,15], was clearly observed (Figure 1A) with isosbestic points at 574, 548 and 517 nm. The absorption at 562 nm was then plotted versus electron equivalents (Figure 1B). It increased up to 1.1 electrons/mol of CDH, and then no further change was observed. In contrast, the absorbance at 449 nm, the isosbestic point of haem [14], did not change during haem reduction, but was decreased by the following 2.1 electrons/mol of CDH. Further addition of dithionite, over 3.2 electrons per CDH, did not cause any spectral changes, except at approx. 315 nm due to excess dithionite, suggesting that fully reduced CDH has acquired 3 electrons, 1 distributed to the haem and 2 to the flavin.

Pre-steady-state kinetic study of flavin and haem reduction

On mixing CDH with cellobiose, the spectrum of the enzyme rapidly changed from oxidized to reduced form over the course of approx. 0.1 s (Figure 2). The spectrum at 0.1 s after mixing with cellobiose had several peaks at 562, 533 and 429 nm, suggesting the reduction of the haem by the substrate. However, unlike the dithionite titration, these peaks appeared after the decrease in absorption around 449 nm, indicating that the flavin reduction proceeded initially, followed by the haem reduction, when cellobiose was used as the electron donor. To observe the pre-steady-state reduction of flavin and haem in CDH, the time courses of the changes of absorption at 449 and 562 nm were monitored by stopped-flow photometry (Figure 3). At 449 nm,

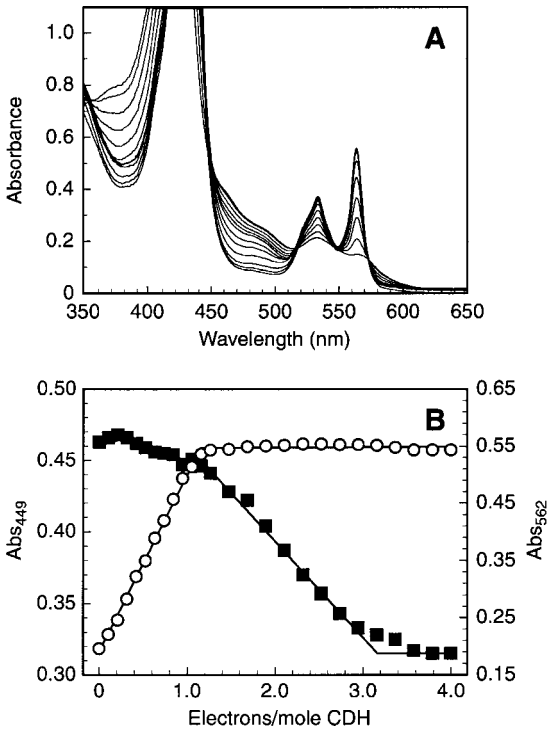

Figure 1 (A) Spectral change and (B) absorption changes at 449 nm (■) and 562 nm (○) during anaerobic titration of CDH

The titration of CDH (17.5 μM) was performed with sodium dithionite in 50 mM Hepes buffer (pH 7.0), at 25 °C.

the absorption decreased immediately after mixing and 62% reduction was observed within 0.025 s (Figure 3A). Since the changes appeared to be biphasic from the time versus $\ln|A - A_\infty|$ plot (Figure 3C), rate constants for the first (k_{obs}^{449F}) and second (k_{obs}^{449S}) phases were estimated as 57.3 ± 1.0 and 17.3 ± 0.4 s^{-1} respectively by fitting the experimental data to a double-exponential function. For haem, the absorption change proceeded with an initial lag, and only 25% of the total change was obtained during 0.025 s (Figure 3B). The time versus $\ln|A - A_\infty|$ plot clearly showed the existence of an initial lag followed by a

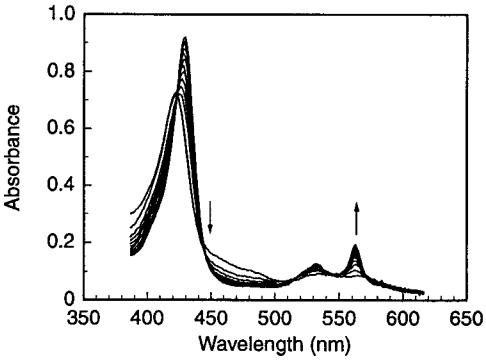

Figure 2 Pre-steady-state spectral changes of CDH upon mixing with cellobiose

CDH and cellobiose (final concentrations of 5 and 500 μM respectively) were mixed in 50 mM sodium acetate (pH 4.5) at 30 °C. Spectra were recorded every 6.5 ms after mixing.

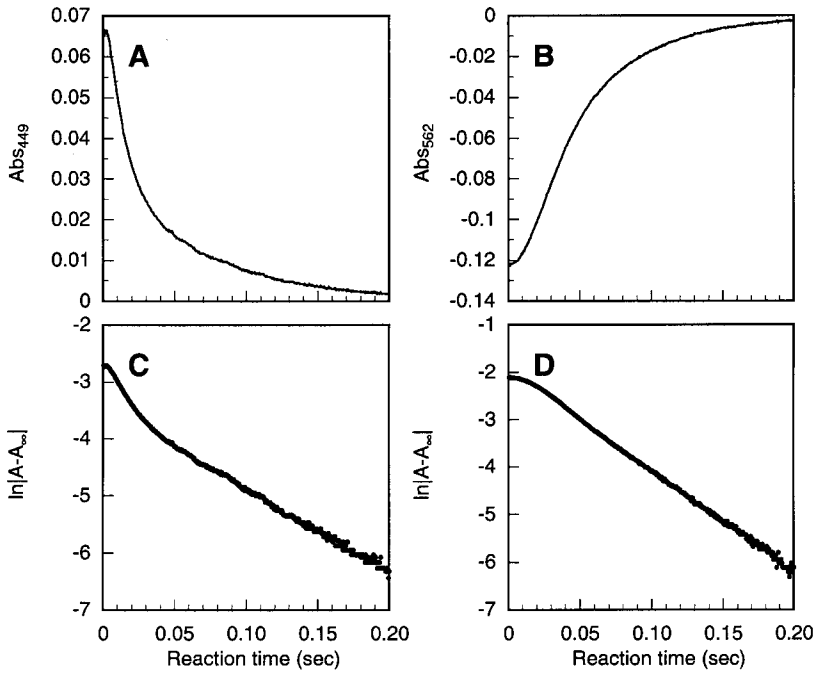

Figure 3 Time courses of absorption at 449 and 562 nm monitored with a stopped-flow spectrophotometer at pH 4.5

CDH and cellobiose (final concentrations of 5 and 500 μM respectively) were mixed in 50 mM sodium acetate (pH 4.5), and the absorption differences at 449 nm (**A**) and 562 nm (**B**) were monitored. (**C**), (**D**) Plots of time versus $\ln|A-A_{\infty}|$.

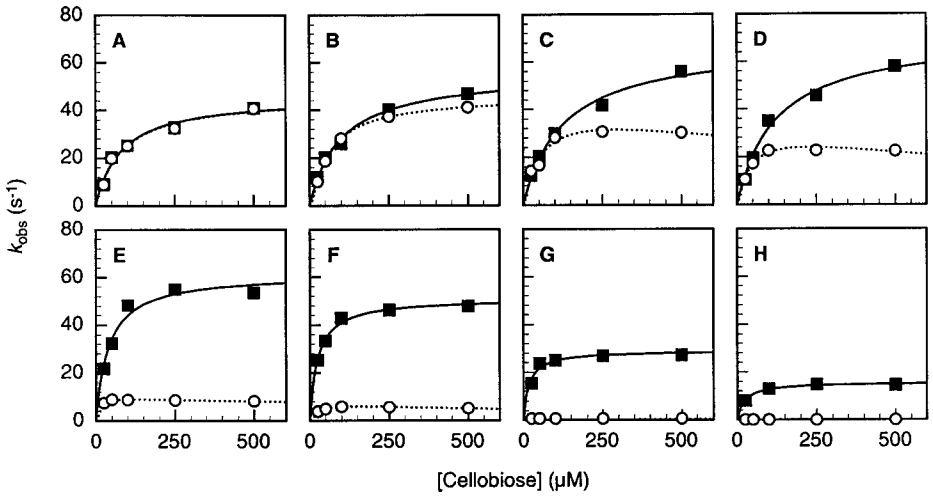

Figure 4 Cellobiose concentration dependence of observed rate constants (k_{obs}) for flavin (■) and haem (○) reduction at pH 3.0 (A), 3.5 (B), 4.0 (C), 4.5 (D), 5.0 (E), 5.5 (F), 6.0 (G), 7.0 (H)

k_{obs} values for both prosthetic groups were obtained with a stopped-flow spectrophotometer in 50 mM buffers of different pH using 25–500 μM cellobiose as a substrate. The fitting of the data was performed as described in the Materials and methods section.

monophasic process (Figure 3D). The rate of the haem reduction (k_{obs}^{562}) was estimated as 17.3 ± 0.2 s^{-1}.

Pre-steady-state kinetic experiments monitoring the flavin and haem reductions were carried out at various cellobiose concentrations and pH values ranging from 3.0 to 7.0 (Figure 4), and limiting values of observed rates (k_{lim}^{449F}, k_{lim}^{449S}, k_{lim}^{562}) were summarized in Table 1. At pH 3.0, the velocity of haem reduction was almost identical with that of flavin reduction, and no significant second phase or lag of haem reduction was observed at any of the cellobiose concentrations tested (Figure 4A). At pH 3.5–4.5, although the reduction rates of both prosthetic groups were identical at low cellobiose concentrations, only haem reduction was affected by substrate inhibition (Figures 4B–4D). Moreover, the apparent second phase of flavin reduction was observed at high concentrations of the substrate (results not shown) and the limiting rates coincided with those of haem reduction within

Table 1 Limiting values of observed rate constants obtained from the time courses of changes of absorption at 449 and 562 nm

Pre-steady-state absorption changes of 5 μM CDH mixed with 500 μM cellobiose were monitored by stopped-flow spectrophotometry in various buffers as described in the Materials and methods section. k_{lim}^{449F} and k_{lim}^{449S} are the limiting rates for the first and second phases at 449 nm respectively, and k_{lim}^{562} is the limiting rate for the change at 562 nm. These rates were estimated by fitting the observed rates as described in the Materials and methods section.

pH	k_{lim}^{449F}	k_{lim}^{449S}	k_{lim}^{562}
3.0	45.9 ± 1.0	n.d.*	45.8 ± 0.8
4.0	67.7 ± 0.9	45.7 ± 0.6	45.2 ± 0.5
5.0	67.5 ± 0.8	16.0 ± 0.6	15.9 ± 0.5
6.0	28.9 ± 0.7	0.5 ± 0.1	0.6 ± 0.1
7.0	14.5 ± 0.4	n.d.*	n.d.†

* No significant second phase was observed.
† No absorption change was observed.

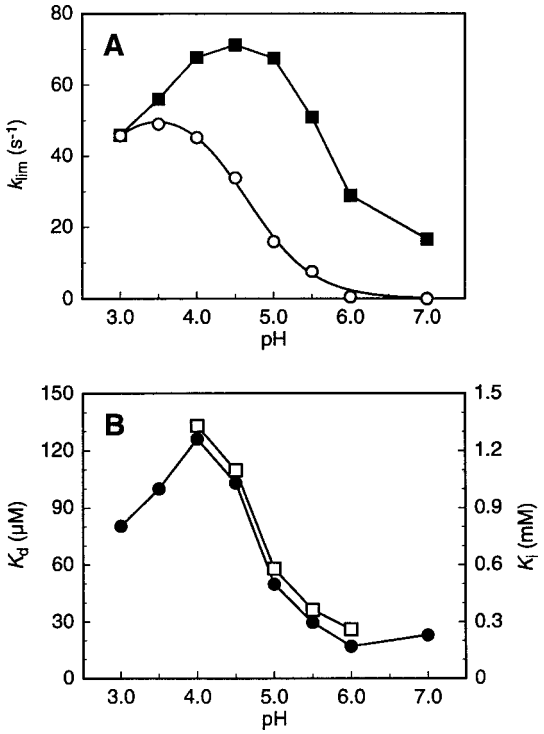

Figure 5 Limiting rate constants k_{lim} for flavin and haem (A) and the dissociation constant K_d and substrate inhibition constant K_i of cellobiose (B) plotted versus pH

The kinetic parameters k_{lim}^{F} (■), k_{lim}^{H} (○), K_d (●) and K_i (□) were obtained from eqns (1) and (2) as described in the Materials and methods section.

the experimental accuracy (Table 1). The inhibition of haem reduction seemed to become stronger with an increase in pH, because k_{lim}^{562} decreased at much lower substrate concentration at higher pH. Above pH 5.0, the haem reduction rate was lower than the flavin reduction rate at all cellobiose concentrations (Figures 4E–4H), and the limiting value became almost zero at pH 6.0, whereas k_{lim}^{449F} was halved (Table 1).

The pH dependence of various kinetic parameters, estimated from Figure 4, was then plotted (Figure 5). k_{lim}^{F} had an optimum at pH 4.5 (71.2 ± 1.0 s^{-1}) and its pH dependence was bell-shaped (Figure 5A). The pH dependence of k_{lim}^{H} also showed a bell-shape, but was somewhat different from that of k_{lim}^{F}; the maximum reduction rate was obtained at pH 3.5 (49.0 ± 0.7 s^{-1}) and was decreased by an increase in pH retaining pK at 4.6. Although both flavin and haem reduction proceeded effectively at pH 4.0, the K_d value at this pH was 126 μM, the highest at any measured pH, indicating that the affinity between substrate and CDH was lowest at this pH. The value decreased with increase in pH, and the maximum affinity of 17.0 μM was obtained at pH 6.0. The K_i values also decreased with increase in pH (1330, 1097, 580, 363, 260 μM at pH 4.0, 4.5, 5.0, 5.5, 6.0 respectively) and the pH dependence showed a tendency similar to that of K_d.

DISCUSSION

As CDH contains two different prosthetic groups, flavin and haem, several redox states may be involved in the catalytic cycle, as is the case for other flavocytochromes [21,22]. The absorption spectra of the prosthetic groups in CDH overlap considerably in the region of 400–500 nm [7]. Monitoring at isosbestic points is therefore an effective way to observe the redox states independently. During dithionite titration, the absorbances at 449 nm (isosbestic point of haem) and 562 nm (isosbestic point of flavin) were completely independent of each other. Moreover, the electron equivalent plots clearly suggest that flavin and haem react as two- and one-electron acceptors respectively. This seems reasonable considering that CDH contains one flavin and one haem in the molecule [6].

When cellobiose was used as a substrate, a rapid decrease in absorption at approx. 450–500 nm was initially observed, followed by increases in α, β and γ bands of haem. The time course of the absorbance change at 449 nm was biphasic and the limiting rate of its second phase was identical with that of the change at 562 nm at various pH values tested, except at pH 3.0. Previous EPR studies of CDH showed that addition of cellobiose increases the flavin radical signal and causes a loss of the haem iron signal [5,16]. Since cellobiose acts as a two-electron donor to the enzyme, one-electron transfer from fully reduced flavin to oxidized haem should cause obligatory formation of flavin semiquinone and reduced haem as shown in the following scheme:

$$F_{ox} + H_{ox} \xrightarrow[+\text{cellobiose}]{1} F_{red} + H_{ox} \xrightarrow{2} F_{sq}H_{red}$$

F_{ox} and F_{red} are oxidized and reduced flavin respectively, F_{sq} is flavin semiquinone and H_{ox} and H_{red} are oxidized and reduced haem. In the above scheme, formation of flavin semiquinone and haem reduction should involve the same electron transfer reaction. Considering the absorption spectra of flavin semiquinones [23], the absorption changes at 449 nm might reflect the flavin semiquinone formation, and thus k_{lim}^{449S} and k_{lim}^{562} are the rates of the same electron transfer process. Since the absorption of haem is strong compared with that of flavin or flavin semiquinone, it is difficult to detect the flavin semiquinone species by spectrophotometry. Although flavin semiquinone could be detected by EPR at the end of the second phase (results not shown), a quantitative EPR study should be performed to identify the semiquinone species.

CDH is known to have optimum activity at approx. pH 4.0 for the steady-state reduction of both quinones and Fe(III)-containing compounds. The initial pre-steady-state kinetic study for the enzyme was performed at pH 6.0 [14] and pH dependence determined [15,24]. Although substrate inhibition of CDH was noted in both the steady-state and pre-steady-state reactions

[5,14,16], no kinetic study has been performed. In the present study, we observed the pre-steady-state reduction of flavin and haem at various pH values, with various cellobiose concentrations. At pH 3.0, the reduction rates of flavin and haem were synchronous at all cellobiose concentrations examined and no lag phase was observed in the absorption changes at 562 nm. This indicates that, at this pH, the electron transfer process from flavin to haem is faster than flavin reduction by the substrate, and the rate-limiting step is flavin reduction at low substrate concentration (pH 3.5–4.5), but changed to haem reduction at high concentrations of cellobiose. The haem reduction was rate-limiting at all substrate concentrations above pH 5.0. Although haem reduction has been considered as a rate-limiting step in the past [14,15,24], the results of this study clearly suggest that the rate-limiting step of the CDH reaction depends on the experimental pH and substrate concentration. The redox potential of F_{ox}/F_{red} of isolated flavin domain decreases with increase in pH from 106 mV (pH 3.0) to -132 mV (pH 7.0) [25], whereas those of haem in CDH at pH 3.0 and 7.0 are 190 and 130 mV respectively [18]. This indicates that the inability of electron transfer from flavin to haem at neutral pH cannot be explained simply by thermodynamic changes of prosthetic groups.

Although flavin reduction had a hyperbolic relation to substrate concentration, only the next electron transfer step was subject to substrate inhibition. In addition, the pH dependence of K_d was identical with that of K_i for the substrate inhibition, indicating that binding of cellobiose to the active site inhibits electron transfer from flavin to haem. Similar substrate inhibition has been observed during the redox reaction of flavocytochrome b_2 [20], and the inhibition was concluded to be due to weak binding of the substrate to the active site when the flavin is in semiquinone form. In CDH, however, the inhibition was observed in the course of the initial electron transfer from flavin to haem, the process in which flavin semiquinone is formed, suggesting that the substrate binds to the reduced type of flavin. It has been reported that the redox potential of flavin in flavocytochrome b_2 was increased in the presence of products, whereas that of haem is not affected [26]. If the release of product is inhibited at high substrate concentration, this might be disadvantageous for haem reduction, not only because of steric inhibition, but also for thermodynamic reasons.

Although the physiological role of CDH is still unclear, the importance of this enzyme in the processes of cellulose degradation and wood invasion has been discussed [27–29]. We have recently succeeded in overexpressing the enzyme, which has similar redox character to the wild-type enzyme, in methylotrophic yeast [30]. Further experiments using site-directed mutants should disclose the electron transfer mechanism of inter-domains in CDH to elucidate its function in cellulose and wood degradation.

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